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Spore Age and Sterilization Affects Germination and Early Gametophyte Development of *Platycerium bifurcatum*

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ABSTRACT.—The effects of spore age and sterilization on spore germination and early gametophyte development were investigated in the fern, *Platycerium bifurcatum*. The highest germination percentage of sterilized spores was obtained with 2–3 month old spores. Further increase in spore age leads to a decline in germination and primary rhizoid initiation. In contrast, spore age had no effect on the germination of unsterilized spores, where maximum response was observed with spores of both storage periods tested, 2 and 14 months. Increasing spore age delays early gametophyte development. In cultures of sterilized spores, this was evident from both a decrease in the length of the primary rhizoid and the decreased number of rhizoids and cells per gametophyte. Although longer primary rhizoids developed from unsterilized spores of both ages, after 10 days other growth parameters of gametophytes were similar or even lower compared to those from sterilized spores.

The viability of spores varies enormously among ferns, ranging from a few days to a few years (reviewed by Miller, 1968). In recent years it has become clear that genetic and certain physiological attributes of spores have to be considered to explain spore longevity (Raghavan, 1989). Storage conditions also are very important for spore viability. Fully hydrated spores showed complete ability to germinate after storage for two years at room temperature (Lindsay et al., 1992). Another possibility for extending the viability of spores is storage at −70°C. This was successfully used for ripe strobili of *Equisetum hyemale* L. (Whittier, 1996). Despite these discoveries, pteridologists generally store fern spores under dry, cold, and dark conditions (Miller and Wagner, 1987; Kadota and Wada, 1989; Grill, 1990; Haupt, 1991; Page et al., 1992; Raghavan, 1993). These storage conditions have for a long time been regarded as favorable. However, it has become evident from many investigations that even under these conditions increasing spore age leads to a decline in viability (Raghavan, 1989).

These experiments concentrated on the effect of spore age on germination, but further gametophyte development has received much less attention. The study of Smith and Robinson (1975) provides valuable information concerning *Polypodium vulgare* L. spores. In their experiments, spores stored for 0–7 years were used, but at intervals of one year only. In another study, spores of *Pteris vittata* L. were stored for 10–100 days and tested at intervals of 20 days (Beri and Bir, 1993). Although different aspects of spore germination and gametophyte development in *Platycerium* have been investigated in detail (Nagmani and Raghavan, 1983; Thentz and Moncousin, 1984; Camloh and Gogala, 1992; Camloh, 1993; Camloh et al., 1996), to the best of our knowledge spore age

effect on developmental processes has never been studied. In the present study, experiments were conducted to determine the effect of spore age (2–14 month old spores were used) and sterilization on germination and particularly on early gametophyte development of the fern *P. bifurcatum*.

MATERIALS AND METHODS

Spores of the fern *Platycerium bifurcatum* (Cav.) C. Chr. were kindly provided by Dr. B. J. Hoshizaki, Univ. of California, Los Angeles. They were collected in September 1991 from mature leaves of a single plant and stored in the dark at 5°C. Spores stored for 2 to 14 months were used in the experiments. They were isolated from sporangial and other debris according to Camloh (1993). The culture method has been described in detail elsewhere (Camloh et al., 1996). Briefly, spores of *P. bifurcatum* sterilized with 70% (v/v) ethanol and 10% (v/v) solution of commercial bleach (4% NaOCl) were sown on the surface of 5 ml modified Knop's solution (Miller and Greany, 1974). Some experiments were also performed with unsterilized spores. The pH of the medium was adjusted to 5.7–5.8 before autoclaving. The media were placed in tubes 24 mm in diameter and covered with plastic caps. Cultures were maintained at 23±2°C, under a 16h photoperiod at 36–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$, provided by cool white lamps (Osram L 65W/20S). Spores were grown in these conditions for 3 to 10 days.

The percentage of germinating spores was evaluated at 3 and 6 days after sowing. The criterion for germination was the breakage of the exine and protrusion of the rhizoid. Samples of at least 100 spores per replicate were examined. The length of the primary rhizoid was measured at three, four, six, and eight days after sowing, and the gametophyte cell number was determined at six, eight, and ten days after sowing. At the end of the experiment, rhizoids were counted. All measurements were made on gametophytes cleaned and stained with acetocarmine-chloral hydrate according to Edwards and Miller (1972) in a microscope fitted with an ocular-micrometer. For the determination of all parameters, except where otherwise specified, at least 25–35 samples were examined per replicate. There were two or three replicates per set of spores of different age. Mean values and standard error (SE), which are represented in the figures as vertical bars, were calculated from the data. The 2×2 Chi-squared test (χ^2) and Student's *t*-test were used for evaluating levels of statistical significance (*P*) between the data obtained with two months old sterilized spores and those obtained with other spore samples.

RESULTS

The effects of spore age and sterilization on germination are shown in Fig. 1. The percentage of germination was determined at three and six days after sowing. Maximum germination of sterilized spores occurred after two to three months of storage, but we must point out the considerable variations in germination obtained in experiments with three months old spores compared to

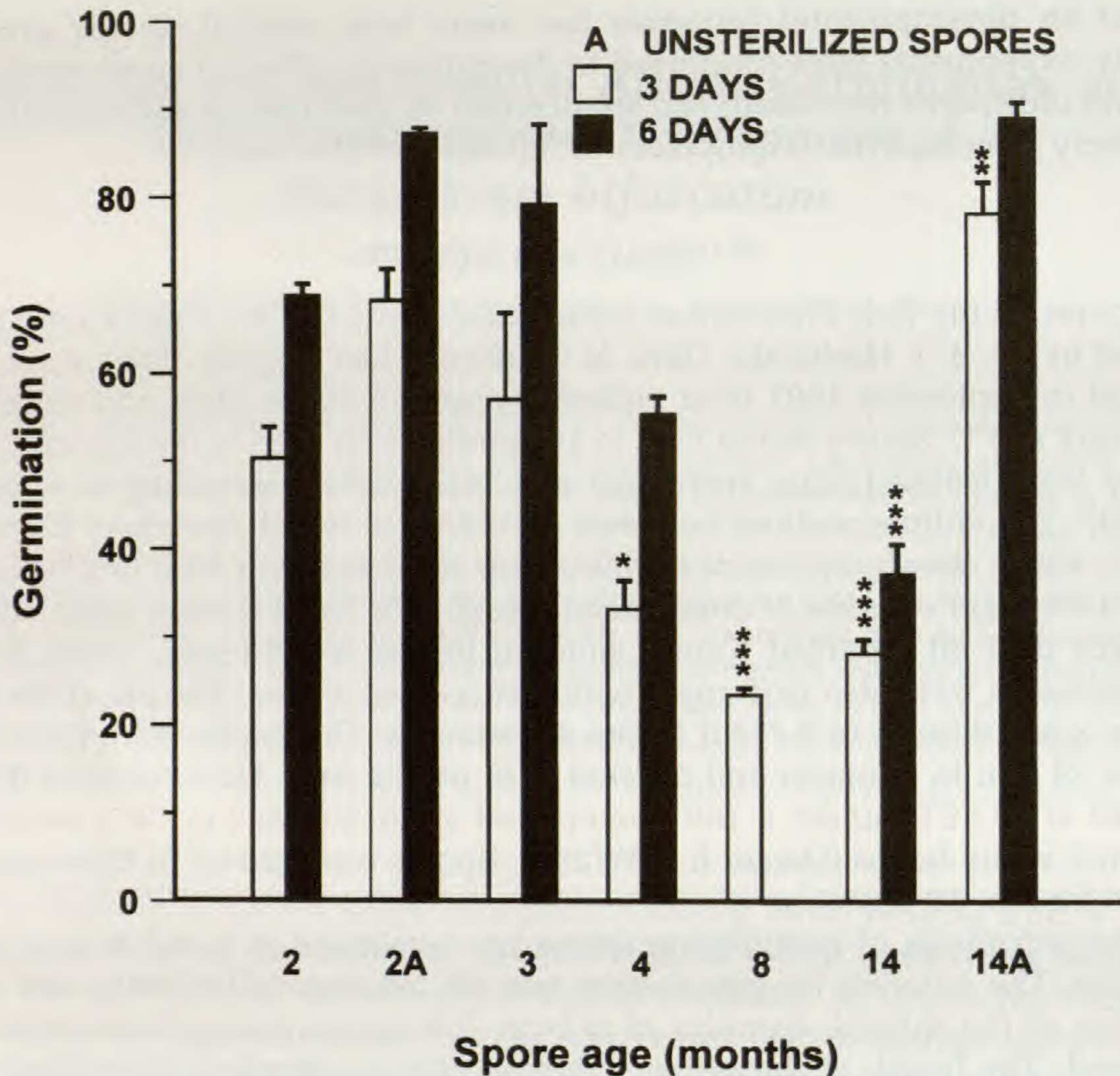

FIG. 1. Effects of spore age and sterilization on germination of *P. bifurcatum* spores. Vertical bars indicate SE. Chi-squared test was used for evaluating the level of statistical significance; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

others. In older spores, substantially decreased germination was observed. Because the criterion for germination was the protrusion of the rhizoid through the exine, this result indicates that spore age affects the process of rhizoid initiation. However, the highest germination percentage was obtained with unsterilized spores. An additional and unexpected result was noted: in contrast to sterilized spores, the germination percentage of unsterilized spores did not decrease with increased storage time (Fig. 1). Although three days after sowing older spores germinated slightly better than younger ones, after six days the germination responses of unsterilized spores of both ages (2 and 14 months) were almost identical. This result suggests that during the sterilization procedure certain changes in spores occurred that resulted in altered germination.

The effects of storage time and sterilization on rhizoid length are shown in Fig. 2. It is evident that as the age of sterilized spores increases, there is a decrease in rhizoid length. This was observed as early as three days after spore sowing. The same age effect also was obtained at four, six, and eight days after sowing. Only when eight month old spores were cultured for eight days was

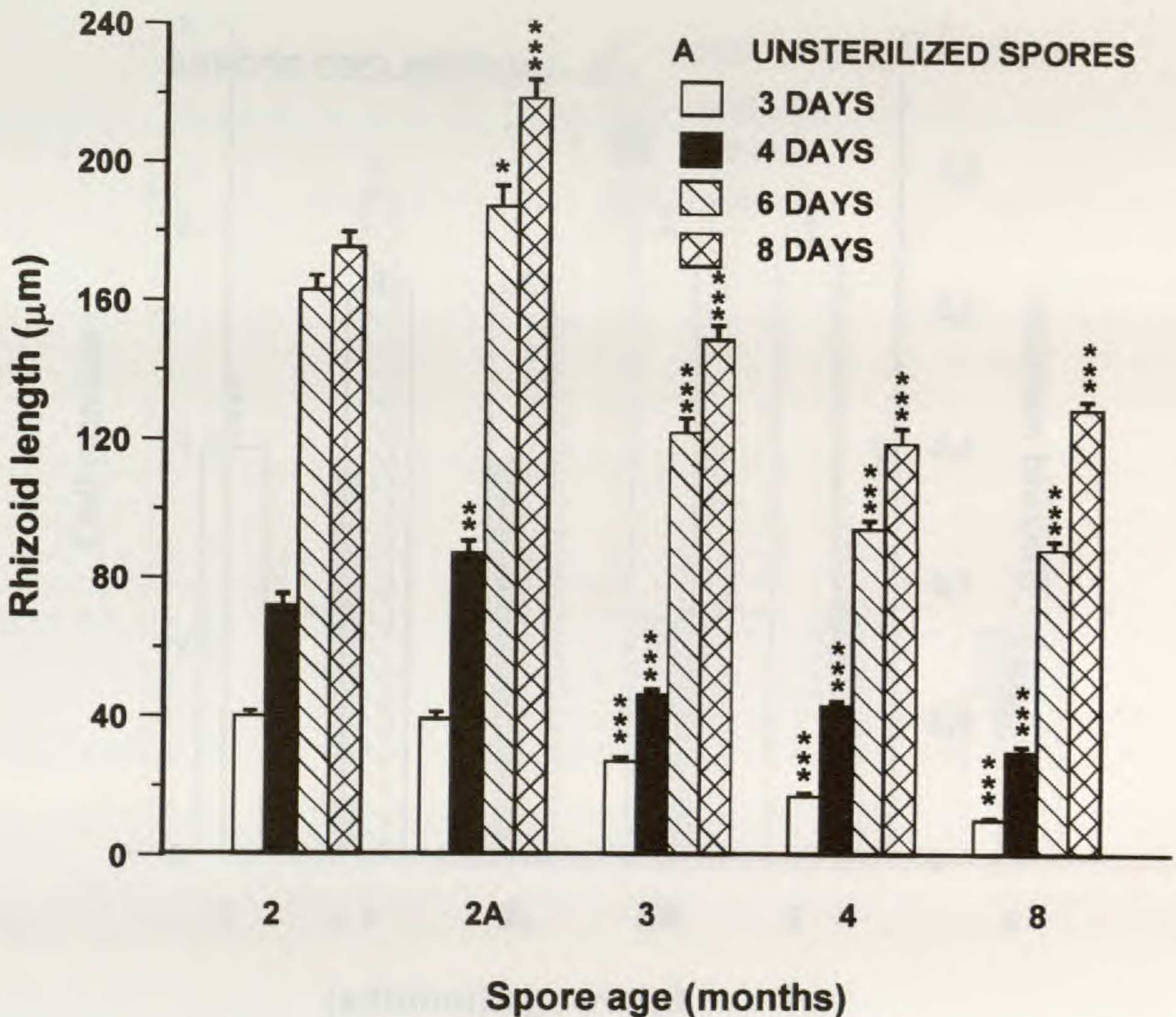

FIG. 2. Effects of spore age and sterilization on the rhizoid length of *P. bifurcatum* gametophytes. Vertical bars indicate SE. Student's *t*-test was used for evaluating the level of statistical significance; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

no further decrease in rhizoid length detected in comparison to four month old spores. Regardless of spore age, the most intensive elongation of the primary rhizoid occurred after between four and six days in culture (Fig. 2). Sterilization also affected rhizoid length. Two month old unsterilized spores cultured for four or more days have longer rhizoids than sterilized spores. This effect was also observed with fourteen month old spores, although their rhizoid length was much smaller in comparison to two month old spores (data not shown).

In addition to rhizoid elongation, spore age also affected rhizoid number (Fig. 3). After ten days of culture, the highest number of rhizoids developed on gametophytes from two month old spores. With increasing spore age the number of rhizoids decrease. The sterilization had no effect on the rhizoid number of two month old spores (Fig. 3). Similarly, in an experiment with fourteen month old spores, no effect of sterilization on rhizoid number was detected (data not shown).

Increasing spore age also leads to a decrease in cell number (Fig. 4). At day

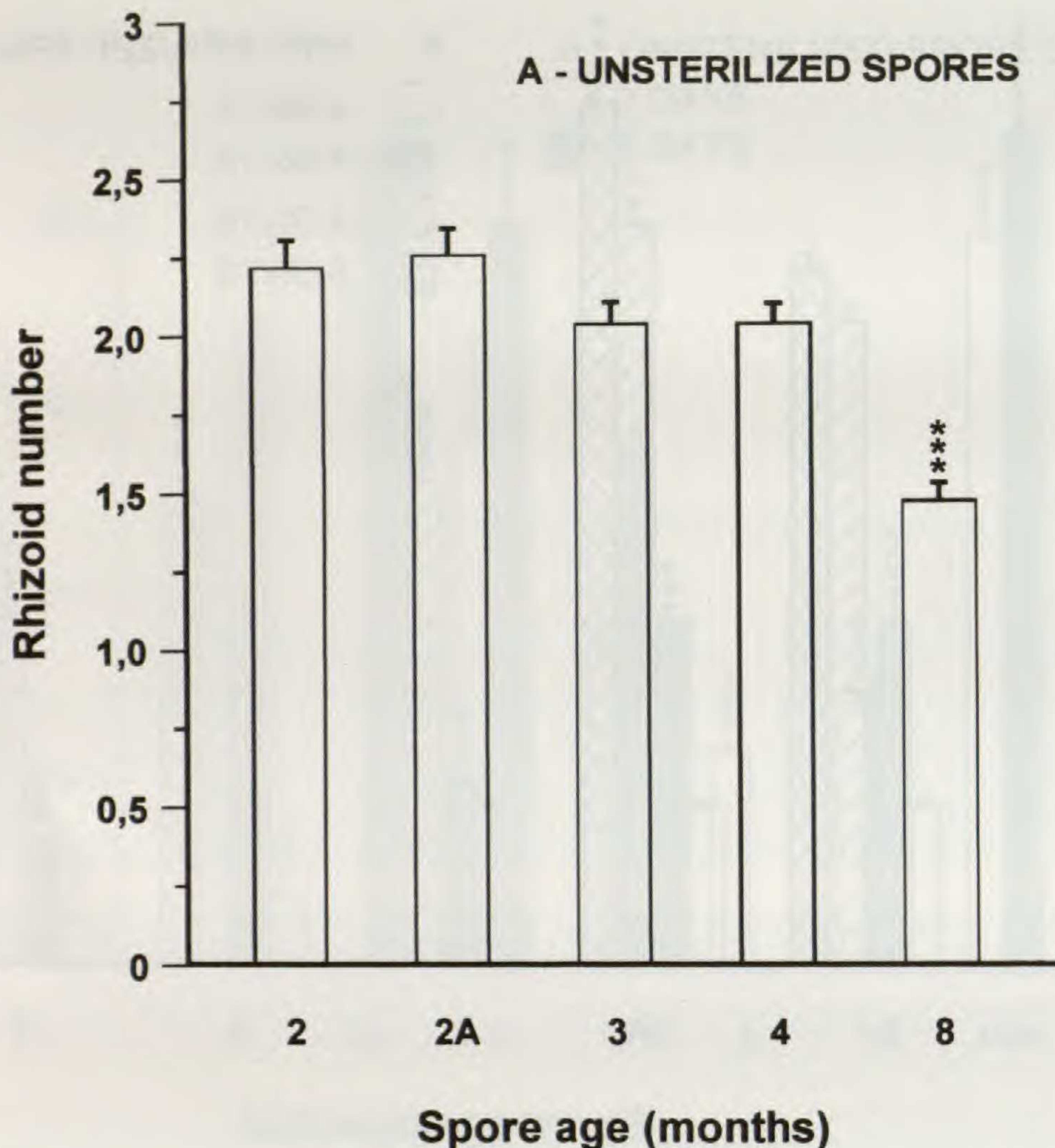

FIG. 3. Effects of spore age and sterilization on the rhizoid number of *P. bifurcatum* gametophytes 10 days after spore sowing. Vertical bars indicate SE. Student's *t*-test was used for evaluating the level of statistical significance; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

six, spore age already has a significant effect, but after two and four more days the decrease in cell number is even more pronounced. After ten days, gametophytes grown from two month old spores have nearly twice as many cells as those grown from older spores. The greatest decrease in cell number occurred between two and three month old spores. Thereafter, increased storage time did not cause a substantial further decrease in cell number. After six and eight days of culture, the cell number of gametophytes grown from sterilized spores is quite comparable with those grown from unsterilized spores. However, after ten days gametophytes with more cells were grown from sterilized spores.

DISCUSSION

Spore age and sterilization affect spore germination and early gametophyte development in *P. bifurcatum*. Sterilized spores gave the highest germination response after two to three months of storage. Three month old spores even

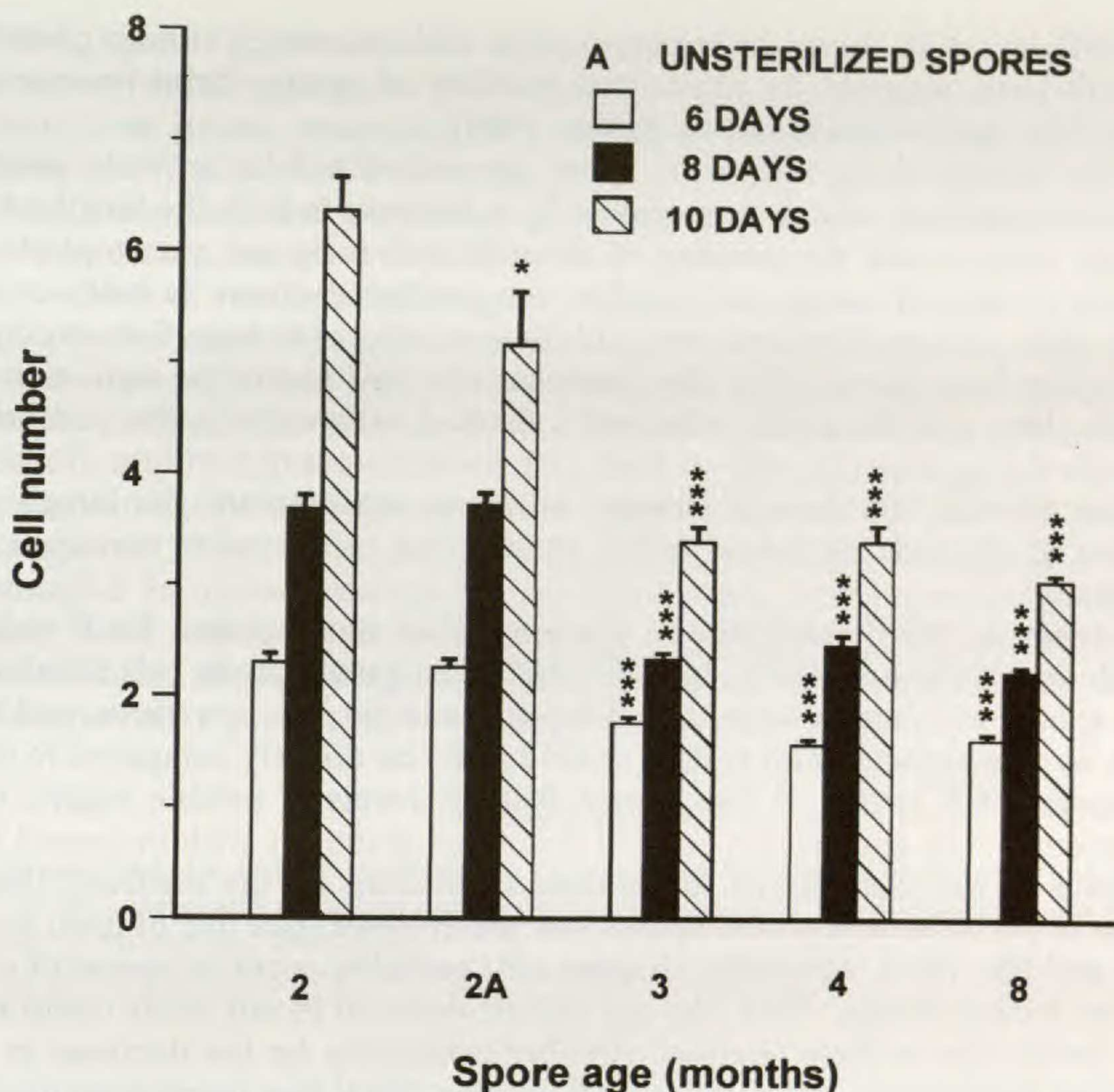

FIG. 4. Effects of spore age and sterilization on the cell number of *P. bifurcatum* gametophytes. Vertical bars indicate SE. Student's *t*-test was used for evaluating the level of statistical significance; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

germinate slightly better than those two months old. Warne and Hickok (1987) reported that freshly collected spores of *Ceratopteris richardii* Brongn. strain Hn-n show a slower rate of germination than older spores. They obtained a maximal germination rate after room temperature storage for several months. Perhaps *Platycerium* spores, like those of *C. richardii* strain Hn-n, also require an after-ripening treatment under dry storage conditions.

When *Platycerium* spores were stored for over three months, a substantial decrease in germination occurred. Similarly, in the fern *Polypodium vulgare,* the rate of germination declines with increasing spore age, but only by 9% after one year of storage (Smith and Robinson, 1975). With spores of *Pteris vittata,* a definite decrease in germination due to storage was reported (Beri and Bir, 1993). They obtained the greatest decrease in germination, 30%, after only 20 days of storage; thereafter germination decreased only slightly, by an additional 10% only after 100 days of storage. In our experiments, germination decreased by 40% between 3 and 14 months of storage. These results, showing

great differences in decreases in germination with increasing storage period in different ferns, support the thesis that viability of spores varies enormously among fern species (reviewed by Miller, 1968).

In the current study, increasing spore age caused a delay in early gametophyte development, which was evident by a decrease in both the length of the primary rhizoid and the number of rhizoids and cells per gametophyte. In contrast to rhizoid length and number, the greatest decrease in cell number, nearly 50%, occurred between two and three months of storage. Gametophytes developing from spores older than three months had nearly the same number of cells. Beri and Bir (1993) obtained a marked difference in the number of rhizoids and protonemal cells in fresh and stored spores of *P. vittata*. However, between 60 and 100 days of storage, as in our experiments, the length and number of rhizoids decreased while protonemal cell number remained unchanged.

Decrease in cell number due to spore age also was reported for *P. vulgare* (Smith and Robinson, 1975). In their study, the gametophyte cell number of fresh spores was compared to that of those stored for one, two, three, and four years, so this experimental system could hardly be directly compared to ours. For spores of *P. vittata*, it was shown that the levels of soluble sugars, total free amino acids, and protein content decreased gradually with increased storage time. It was also shown that during incubation on the medium, the increase in the volume of stored spores was much lower than that of fresh spores (Beri and Bir, 1993). Metabolic changes also probably occur in spores of other species during storage. Thus, the age effects observed in our study could be at least partly due to these changes. Another possibility for the decrease in germination that was reported earlier (Beri and Bir, 1993) may be changes in water content.

In a substantial majority of reports concerning fern spores, experiments were performed with sterilized spores only. However, in our work unsterilized spores also were used. The best germination and rhizoid elongation were obtained with these unsterilized spores (Camloh, 1993, and present results). In our experiments with unsterilized spores, a result was obtained to which special attention should be paid. With an increasing storage period, the germination percentage of unsterilized spores, in contrast to that of sterilized spores, did not decrease. It is known that environmental factors during spore ripening or slight changes in experimental conditions can affect germination (Haupt et al., 1988). However, because the same spore sample was used throughout the experiments and sterilized and unsterilized spores were always tested at the same time, our results could not have been influenced by these factors.

The explanation of this observation could lie in changes that might occur in spores during the sterilization procedure. In our experiments, spores were sterilized with ethanol and a solution of commercial bleach (see Materials and Methods). Both of these substances could damage spores. Various alcohols (Miller, 1987; Vogelmann and Miller, 1981) and also a dilute solution of NaOCl (Howland and Boyd, 1974) delay or inhibit the germination of spores. Treatment of spores with NaOCl was used to remove the exine from *Onoclea sen-*

sibilis L. spores (Vogelmann and Miller, 1980; Miller et al., 1983). Furthermore, the use of NaOCl appears to be a relatively non-specific method of removing cations from spores, especially Ca^{2+}, which is essential for spore germination (Miller and Wagner, 1987). Changes in Ca^{2+} in plants have profound effects in cellular functions, and a number of cellular processes have been identified that depend on changes in cytosolic Ca^{2+} (reviewed by Bush, 1993). The different effects of sterilization on spores of different ages indirectly showed that some physiological changes occurred in spores during storage. An explanation for these age-dependent effects of sterilization might be connected with changes in the permeability of spore walls. If the wall permeability increases as the storage period lengthens, this would result in greater detrimental effects of sterilization agents in older spores; for example, more Ca^{2+} could be removed from spores. Thus, spore germination and gametophyte development would be delayed. In unsterilized spores, the higher spore wall permeability of older spores could lead to faster imbibition and germination. However, other metabolic changes, already discussed, that occur in spores during storage resulted in delayed early gametophyte development in older spores.

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